ESO10-53 peptide, whereas CTL clone 5 strongly recognized the ESO10-53 peptide when pulsed onto HLA-A31 positive 586EBV B cells. TIL1244 recognized the TRP197-205 peptide derived from TRP-2, 586EBV B cells alone or pulsed with the ORF3P peptide from an alternative reading frame of TRP1 were negative controls. 397mel is an HLA-A31-negative, NY-ESO-1-positive tumor line.

Figure 7A-7C. An alternative open reading frame of the *NY-ESO-1* gene and antigenic peptides recognized by CTL. Figure 7A. Identification of antigenic peptides from the ORF2. Thirty peptides were synthesized based on all potential ORFs and screened. Representative data are shown here. Seven peptides derived from ORF2 (see Fig. 3A) were pulsed on HLA-A31-positive 1510EBV B cells and tested for T cell recognition based on GM-CSF release. (A) insert 1510EBV alone was used as a negative control. Figure 7B. 1510EBV B cells were labeled with chromium for 2 h. The ESORF2-10-18 peptide was then pulsed on the chromium-labeled 1500EBV (solid square) and HLA-A31-negative 1102EBV (open circle) at different concentrations. 1510EBV pulsed with ESO10-53, which was recognized by CTL clone 5, was used for the specificity control (solid triangle). After peptide incubation and three washes, cytolysis of target cells by CTL-clone 2 was determined in a 4-h chromium release assay at an E:T ratio of 20:1. Figure 7C. Several tumor lines and fresh breast tumors were tested for recognition by CTL clone 2 to determine whether the ORF2 is translated in different tumors. 1510EBV B cells pulsed with ESO10-53 ASGPGGGAPR (SEQ ID NO:25), ORF2-10-18 LAAQERRVPR (SEQ ID NO:47) or alone were included to evaluate the reactivity and specificity of CTL clone 2 and 5. Expression of HLA-A31 on the tumor cells is indicated.

Detailed Description of the Invention

The present invention encompasses cancer peptides, tumor antigen and portion, derivatives or variants thereof which are immunologically recognized by T lymphocytes of the immune system. The present invention further encompasses the antigenic cancer epitope(s) which are contained in the cancer peptides or tumor antigen. The antigenic cancer epitope specifically causes a cellular mediated immune

insert (A) for Page 7

(GAMLAAQER, SEQ ID NO: 123;

AMLAAQERR, SEQ ID NO: 124;

PGAQGQQGPR, SEQ ID NO: 125;

AAQERRVPR, SEQ ID NO: 46;

LAAQERRVPR, SEQ ID NO: 47;

GPRGREEAPR; SEQ ID NO: 126; and

APRGVRMAAR; SEQ ID NO: 127)

Table 4
HLA peptide motif search results

User Parameters and Scoring Information	
Method selected to limit number of results	explicit number
Number of results requested	30
HLA molecule type selected	A-3101
Length selected for subsequences to be scored	10
Echoing mode selected for input sequence	Y
Echoing format	numbered lines
Length of user's input peptide sequence	180
Number subsequence scores calculated	171
Number of top-scoring subsequences reported back in scoring output table	30

Scoring Results			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	127	TVSGnILTIR	4.000 Seq. ID No. 84
2	134	TIRLtAADHR	2.000 Seq. ID No. 85
3	97	ATPMeAELAR	2.000 Seq. ID No. 115
4	170	FLAQpPSGQR	2.000 Seq. ID No. 116
5	98	TPMEaELARR	1.200 Seq. ID No. 117
6	77	RCGArGPESR	0.600 Seq. ID No. 118
7	68	AASG1NGCCR	0.200 Seq. ID No. 119
8	171	LAQPpSGQRR	0.200 Seq. ID No. 120
9	163	TQCF1PVFLA	0.120 Seq. ID No. 86
10	153	LQQLsLLMWI	0.080 Seq. ID No. 87
11	115	PLPVpGVLLK	0.080 Seq. ID No. 88
12	152	CLQQ1SLLMW	0.080 Seq. ID No. 89
13	131	NILTiRLTAA	0.080 Seq. ID No. 90
14	126	FTVSgNILTI	0.080 Seq. ID No. 91
15	43	RGPRgAGAAR	0.060 Seq. ID No. 121
16	158	LLMWiTQCFL	0.060 Seq. ID No. 92
17	87	LLEFyLAMPF	0.040 Seq. ID No. 93
18	161	WITQcFLPVF	0.040 Seq. ID No. 94
19	157	SLLMwITQCF	0.040 Seq. ID No. 95
20	93	AMPFaTPMEA	0.040 Seq. ID No. 96
21	72	LNGCcRCGAR	0.040 Seq ID No. 122
22	154	QQLS1LMWIT	0.040 Seq. ID No. 97
23	86	RLLEfYLAMP	0.024 Seq. ID No. 98
24	143	RQLQ1SISSC	0.024 Seq. ID No. 99
25	71	GLNGcCRCGA	0.020 Seq. ID No. 100
26	91	YLAMpFATPM	0.020 Seq. ID No. 101
27	22	GIPDgPGGNA	0.020 Seq. ID No. 102
28	53	ASGPgGGAPR	0.020 Seq. ID No. 25
29	144	QLQLsISSCL	0.020 Seq. ID No. 103
30	133	LTIR1TAADH	0.020 Seq. ID No. 104

Table 5
HLA peptide motif search results

User Parameters and Scoring Information	
Method selected to limit number of results	explicit number
Number of results requested	30
HLA molecule type selected	A-3101
Length selected for subsequences to be scored	9
Echoing mode selected for input sequence	Y
Echoing format	numbered lines
Length of user's input peptide sequence	181
Number subsequence scores calculated	173
Number of top-scoring subsequences reported back in scoring output table	30

Scoring Results			
Rank	Start Position	Subsequence Residue Listing	Score (Estimate of Half Time of Disassociation of a Molecule Containing This Subsequence)
1	172	AQPPSGQRR	2.000 Seq. ID No. 107
2	98	TPMEAELAR	1.200 Seq. ID No. 108
3	99	PMEAELARR	0.400 Seq. ID No. 109
4	86	RLLEFYLAM	0.240 Seq. ID No. 63
5	38	GATGGRGPR	0.200 Seq. ID No. 110
6	44	GPRGAGAAR	0.200 Seq. ID No. 111
7	171	LAQPPSGQR	0.200 Seq. ID No. 112
8	154	QQLSLLMWI	0.160 Seq. ID No. 64
9	116	LPVPGVLLK	0.160 Seq. ID No. 65
10	120	GVLLKEFTV	0.120 Seq. ID No. 66
11	131	NILTIRLTA	0.080 Seq. ID No. 67
12	161	WITQCFLPV	0.080 Seq. ID No. 68
13	127	TVSGNILTI	0.080 Seq. ID No. 69
14	153	LQQLSLLMW	0.080 Seq. ID No. 70
15	159	LMWITQCFL	0.060 Seq. ID No. 71
16	158	LLMWITQCF	0.060 Seq. ID No. 72
17	132	ILTIRLTAA	0.040 Seq. ID No. 73
18	148	SISSCLQQL	0.040 Seq. ID No. 74
19	128	VSGNILTIR	0.040 Seq. ID No. 113
20	145	LQLSISSCL	0.040 Seq. ID No. 75
21	135	IRLTAADHR	0.040 Seq ID No. 114
22	152	CLQQLSLLM	0.040 Seq. ID No. 76
23	110	AQDAPPLPV	0.040 Seq. ID No. 77
24	164	QCFLPVFLA	0.036 Seq. ID No. 78
25	143	RQLQLSISS	0.024 Seq. ID No. 79
26	108	SLAQDAPPL	0.020 Seq. ID No. 80
27	73	NGCCRCGAR	0.020 Seq. ID No. 81
28	134	TIRLTAADH	0.020 Seq. ID No. 82
29	54	SGPGGGAPR	0.020 Seq. ID No. 14
30	69	ASGLNGCCR	0.020 Seq. ID No. 83

Peptides were synthesized based on the peptide binding motif for HLA-A31 (hydrophobic residues at position 2 and positively charged residues at position 9) (Rammensee et al. 1995, Immunogenetics 41:178-228) and tested for reactivity with CTL clone 5.

These peptides were pulsed onto HLA-A31-positive 1510EBV B cells and tested for their ability to stimulate cytokine release by CTL clone 5. As shown in Table 6, the 10-mer peptide ESO10-53 (ASGPGGGAPR) (SEQ ID NO: 25), starting at position 53 of the NY-ESO-1 protein was strongly recognized by CTL clone 5, while the overlapping 9-mer peptides, ESO9-54 as well as ESO10-127, were weakly recognized when pulsed onto 1510EVB B cells. CTL clone 10 recognized the same peptide as CTL clone 5 (data not shown). Interestingly, CTL clone 2 did not recognize any of these peptides (Table 6), even though it recognized 586mel and COS-7 transfected with NY-ESO-1 (see below). The reactivity of CTL clone 5 was undetectable when either the ESO9-54 or the ESO10-127 peptides were used at concentrations below 100 nM to sensitize EBV cells.

observed at an E:T ratio of 2.5:1 E:T. CTL clone did not lyse either 586EBV or 1515EBV B cells alone or pulsed with an irrelevant peptide, nor did it lyse the HLA-A31-negative T2 cells pulsed with the ESO10-53 peptide (Fig. 5C).

Next, it was tested whether T cell recognition of the 10-mer peptide could be improved by substituting amino acids at anchor residues. A number of synthetic peptides with modification at residues 1, 2 and 10 were made and tested for recognition by CTL clone 5 when pulsed onto 586EBV B cells (Table 7). The modified 10-mer peptides with a substitution at position 2 derived from the wild-type ASGPGGGAPR (SEQ ID NO: 25) were still recognized by CTL clone 5 when pulsed on 586EBV B cells. The reactivity of peptides containing a substitution of either Ala, Ile, Leu or Val at position 2 was lower than that of the wild-type peptide, while one peptide containing a substitution of Thr for Ser at position 2 resulted in a slightly higher reactivity than the wild-type ESO10-53 peptide. In contrast, peptides containing substitutions of Arg with Lys or His completely lost their ability to stimulate T cells, suggesting that the Arg at the C-terminus of the ESO10-53 peptide represents a critical anchor residue. Peptides with a substitution at position 1 were recognized poorly or not recognized at all by CTL clone 5 (Table 7). These results indicate that the ESO-53 peptide, ASGPGGGAPR (SEQ ID NO: 25) represents the best peptide for T cell recognition.

Example 12

Antigenic Peptides Derived From An Alternative Open Reading Frame

Two additional CTL clones, clones 2 and 14, appeared to recognize 586mel as well as COS-7 cells transfected with NY-ESO-1 and HLA-A31 cDNA, but failed to recognize the ESO 10-53 peptide (Fig. 6A-6H). CTL clone 5 and TIL1244 were used for the specificity controls. Additional experiments showed that CTL clone 2 did not respond to any of 19 other peptides containing the HLA-A31 binding motif derived from the normal open reading frame of NY-ESO-1 (Table 6). To test the hypothesis that CTL may recognize a peptide from a gene product translated from an alternative open reading frame of the same gene, synthetic peptides were made with HLA-A31

binding motif on the basis of amino acid sequence predicted from the second open reading frames (ORF2) (Fig. 3A). Strikingly, CTL clone 2 recognized ESORF2-9-19 (AAQERRVPR) (SEQ ID NO: 46) as well as the overlapping ESORF2-10-18 (LAAQERRVPR) (SEQ ID NO: 47) peptides when pulsed onto 1510EBV B cells. Representative data for CTL clone 2 is shown in Fig. 7A. CTL clone 14 recognized the same peptides as CTL clone 2 (Data not shown). These results suggest that CTL clones 2 and 14 recognized an antigenic peptide derived from the ORF2 (Fig. 3A). A protein database search revealed that the 58 amino acid protein of ORF2 has a 52% similarity to the chain A of glutamate dehydrogenase in a 25 amino acid region (34). Peptide titration experiments demonstrated that CTL clone 2 was capable of lysing 1510EBV pulsed with ESORF2-10-18 (LAAQERRVPR) (SEQ ID NO: 47) at relatively low concentrations of peptide, but failed to lyse 1510EBV pulsed with ESO10-53 or HLA-A31-negative 1102EBV pulsed with ESORF2-10-18 (Fig. 7B). In addition, CTL clone 2 also recognized overlapping 11mer, 12-mer, and 13-mer peptides with amino acid extensions at the N terminus of the ESORF2-10-18 peptide at relatively high concentrations (data not shown).

Additional experiments were carried out to determine whether CTL clones recognize the ORF2 gene product of the NY-ESO-1 in other tumor types. As shown in Fig. 7C, the recognition pattern of CTL clone 2 was similar to that of CTL clone 5 on tumor cells. CTL clone 2 recognized HLA-A31 positive fresh 1315Br and 1295Br breast tumors as well as 586mel and 1388mel, but did not recognize HLA-A31 negative fresh 1411Br breast tumor, 397mel, nor the HLA-A31 negative 1295 fibroblast. Although 1353mel expresses HLA-A31, neither CTL clone 2 nor clone 5 responded to 1353mel because 1353mel is NY-ESO-1 negative tumor. As previously demonstrated, CTL clone 5 recognized the ESO10-53 ASGPGGGAPR peptide (SEQ ID NO: 25) and CTL clone 2 recognized the ORF2-10-18 LAAQERRVPR peptide (SEQ ID NO: 47) derived from the ORF2 following incubation with 1510EBV B cells (Fig. 7C). These results strongly suggest that the ORF2 gene product was translated, processed and presented in melanoma as well as breast tumors. Therefore, *NY-ESO-1* encodes two different